



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

(11) Publication number:

0 226 288  
A2

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number: 86307747.5

(51) Int.Cl.<sup>4</sup>: C 12 Q 1/68

(22) Date of filing: 08.10.86

C 12 N 15/00, C 12 N 7/00  
C 12 P 19/34

(30) Priority: 09.10.85 US 785798  
29.05.86 US 868351

(72) Inventor: Braman, Jeffrey C.  
Alberta Drive Hudson  
Massachusetts 01749(US)

(43) Date of publication of application:  
24.06.87 Bulletin 87/26

(72) Inventor: Buchwald, Manuel  
353 Riverdale Avenue Toronto  
Ontario M6J184(CA)

(84) Designated Contracting States:  
AT BE CH DE ES FR GB GR IT LI LU NL SE

(72) Inventor: Kelier, Helen-Donis  
80 Park Street Brookline  
Massachusetts 02146(US)

(71) Applicant: COLLABORATIVE RESEARCH INC.  
Two Oak Park  
Bedford Massachusetts 01730(US)

(72) Inventor: Knowlton, Robert G.  
30 Shirley Street Lexington  
Massachusetts 02173(US)

(72) Inventor: Barker, David F.  
45 Kilgore Avenue Medford  
Massachusetts 02155(US)

(72) Inventor: Schumm, James W.  
27 Moningside Avenue Natick  
Massachusetts 01760(US)

(72) Inventor: Tsui, Lap-Chee  
170, Elisabeth Street, Hospital Children  
Toronto/Ontario M5G 1X8(CA)

(74) Representative: Shipley, Warwick Grenville  
Michael et al,  
VENNER, SHIPLEY & CO. 368 City Road  
London EC1V 2QA(GB)

(54) Means and method of testing for cystic fibrosis based on genetic linkage.

(57) The present invention provides a method of determining presence or absence of one or more cystic fibrosis alleles in a DNA sample taken from an individual having a family genetic background in which family, DNA of ancestors in the family line contains identified cystic fibrosis causing allele genes, said method comprising,

restriction digesting a DNA sample to be tested, which DNA sample is from an individual having a family genetic background in which family ancestors of the family have DNA which contains identified cystic fibrosis allele;

apply a probe to said DNA sample which probe hybridizes within less than 50 centimorgans of a cystic fibrosis gene in said family DNA,

and determining whether or not hybridization has taken place in a manner consistent with the presence of one or more cystic fibrosis alleles in said sample.

EP 0 226 288 A2

MEANS AND METHOD OF TESTING FOR CYSTIC  
FIBROSIS BASED ON GENETIC LINKAGE

Cystic Fibrosis (CF) is the most common lethal genetic disease present in United States populations with a frequency of about 1 in 2000 live births in the United States. The allele frequency in the population is almost 1 in 20 individuals. It is classified lethal since clinical features of the ailment are often sufficiently serious to limit life expectancy to the second or third decade of life. The course of the disease can be quite variable among patients but early diagnosis and improved therapies have led to an increase in life expectancy.

Cystic Fibrosis is a genetic disease that is transmitted from parents to offspring as an autosomal recessive trait. An autosomal recessive trait is one in which each parent contributes a mutant or defective allele to the child who then

-2-

expresses the disorder. The parents of a Cystic Fibrosis offspring can be afflicted wth cystic Fibrosis or more usually are carriers of the gene defect but do not show any clinical symptoms of the disease. Due to the lack of any diagnostic test, the parents are unaware of their CF carrier status until a CF child is produced.

Although Cystic Fibrosis is generally acknowledged as a single gene disorder transmitted as an autosomal recessive, significant differences in the severity of the disorder among individuals suggest either genetic heterogeneity (one parent alone can transmit a dominant CF gene) or that some mutant alleles are more debilitating than others. Alternatively, other researchers argue that several different loci (genes) could produce the phenotype (disease) classified as CF.

Clinically, CF is a generalized disease of exocrine glands that can cause body problems having symptoms which include pancreatic insufficiency, pulmonary obstruction, respiratory tract infection and other severe disorders.

Diagnosis of CF is most commonly limited to individuals who clearly display symptoms of the disease. An initial indication of CF is usually suspected from characteristic pulmonary and gastrointestinal findings, or a positive family

- 3 -

history. The current accepted confirmation of diagnosis of CF is documentation of an elevated sweat electrolyte concentration. The only acceptable sweat test method is the quantitative pilocarpine iontophoresis sweat test. This test has not been useful as a general screen because of the high cost and difficulty in obtaining an adequate quantity of sweat from newborns.

A number of homozygote CF afflicted persons and CF carrier (heterozygote) screening tests other than the sweat test have been proposed over the past few years but none have been instituted in large scale screening services. The most widely used postnatal screening tests are those based on the presence of increased amounts of albumin or protein in meconium (mucous like secretions found in the intestines) and which reflect a reduction or absence of pancreatic proteolytic enzymes. Unfortunately, these tests often do not detect the 15-20 percent of homozygotes with normal pancreatic function. Additional problems include occasional false negative values in persons with pancreatic insufficiency and false positive values in the presence of prematurity or melena. The simultaneous measurement of beta-D-fucosidase and lactase in meconium may increase the specificity of the test. A recently proposed screening method uses dried blood spot assay for immunoresortive trypsin. However, an

-4-

extensive evaluation project including screening of over 90,000 newborns found the predictive value of this latter test to be only 17% (Hammond et al., 1984).

Other tests currently being carried out including bioassays for sodium transport inhibitory factors and mucociliary factors have been difficult to standardize and are not useful for heterozygote screening. Further, studies that initially showed increased steroid and ouabain resistance or abnormal sodium transport in fibroblasts from CF homozygotes and heterozygotes were later shown to be not reproducible. A prenatal diagnosis testing scheme utilizing monoclonal antibodies to three isoenzymes of alkaline phosphatase from amniotic fluid of second trimester pregnancies has been proposed. Low enzyme levels are scored as the CF phenotype. The false positive values were listed as 6% and 23/26 CF cases were found to have 1/2 median value for the corresponding week of gestation (Brock et al., 1985). This proposed test awaits large scale evaluation and has been questioned for its lack of precision.

Recently a serum protein activity (paraoxonase or PON) has been shown to be genetically linked to cystic fibrosis by studies of coinheritance of the marker in families segregating CF (Eiberg, H., Schmiegelow, K., Tsui, L.-C., Neiburhr, E., Phelan,

-5-

P.D., Williamson, R., Warwick, W., Schwartz, M., Koch, C. and Mohr, J. *Cytogenet. Cell Genet.*, in press, and Schmeigelow, K., Eiberg, H., Buchwald, M., Tsui, L.-C., Phelan, P.D., Williamson, R., Warwick, W., Neiburhr, E., Mohr, J., Schwartz, M. and Koch, C. *Lancet*, in press). However, it is unlikely that this enzyme activity will furnish a diagnostic test because prenatal samples cannot be analyzed. While many biochemical parameters have been analyzed, no generally accepted reproducible prenatal test is available for homozygotes nor are there any generally accepted heterozygote screening tests available.

In addition to the lack of reliable postnatal homozygote diagnostic tests, there are no reliable prenatal homozygote tests currently available and no satisfactory heterozygote analysis (carrier testing) can be done at the present time. Diagnosis in advance of symptoms would clearly be of benefit for treatment and as a means of providing information for genetic counseling.

Because of the lack of availability of heterozygote testing and prenatal diagnosis, probabilities based on mendelian genetics are the only information that can be reliably given to families with a positive family history of CF. For example, parents who have given birth to a CF child are obligate heterozygotes with one out of four offspring inheriting both mutant alleles and

-6-

developing CF. With the latter information, parents will only know that with each new birth the risk factor is 25% for CF and 50% for carrier status. Diagnostic tests based on mendelian genetics are not able to give a parent high probability whether the at risk individual is a carrier, has the disease or is free of the disease gene.

Recently gene linkage testing for a dominant genetic trait, i.e. Huntington's Chorea has been carried out by others using the DNA probes in hybridization test steps. However, there has been no accepted linkage test used for determining presence or absence of autosomal recessive traits such as disease and specifically cystic fibrosis disease.

It is an object of the present invention to provide a method for detecting autosomal recessive trait genes.

It is a further object of the present invention to provide a detection method that utilizes genetic linkage.

Another object of the present invention is to provide a cystic fibrosis disease diagnostic test which could determine whether an individual is a carrier, has the disease or is free of the disease

- 7 -

gene.

It is an additional object of the present invention to provide a means and method for identifying whether or not a cystic fibrosis gene is present in a DNA sample.

Another object of the present invention is to provide a method for linkage to the cystic fibrosis gene using restriction fragment length polymorphism (RFLP) probes.

According to the invention, a DNA probe is provided which has a PIC value of at least 0.58. The probe hybridizes within less than 50 centimorgans of a cystic fibrosis gene locus and preferably within 10 centimorgans of the locus. The probe can be Lam4-917.

In a method of identifying whether or not an autosomal recessive trait gene is present in a DNA sample taken from an individual, a probe is applied under hybridizing conditions to a DNA sample which probe identifies an autosomal recessive trait gene locus specific to an autosomal recessive trait gene in the DNA if present. It is then determined whether or not the probe has hybridized to the DNA. Preferably the gene is a gene which causes disease in humans as for example a cystic fibrosis gene. The hybridization is examined to determine if it has taken place in a manner consistent with the presence

-8-

of one or more cystic fibrosis alleles. This is done by comparing results of hybridization with the results obtained from DNA of the parents of the individual from whom the sample to be tested was taken, so that hybridization at similar positions on a solid support with respect to each parent, indicates the presence or absence of a cystic fibrosis allele obtained from each parent in the DNA tested.

Preferably a method of determining the presence or absence of one or more cystic fibrosis alleles in a DNA sample taken from an individual having a family genetic background in which family, DNA of ancestors in the family line contain cystic fibrosis genes, comprises obtaining a DNA sample from the individual, restriction digesting the DNA sample to be tested and applying a probe to the DNA sample which probe hybridizes within less than 50 centimorgans of a cystic fibrosis gene in the family DNA. There is then a determination made as to whether or not hybridization has taken place in a manner consistent with the presence of one or more cystic fibrosis alleles in the sample.

It is a feature of this invention that a test can be carried out on a pre-born fetus, an individual showing no symptoms of the autosomal recessive trait or any individual so long as there

-9-

is a family of ancestor's DNA available for comparison hybridization with a selected DNA probe which hybridizes with an autosomal recessive trait gene in that family. In most instances diagnostic tests have a high degree of accuracy when positive and a higher degree of accuracy when negative than hitherto used diagnostic procedures. The testing can be carried out with minimum inconvenience to the parties involved and with small samples of DNA taken from a variety of body locations.

BRIEF DESCRIPTION OF THE TABLES

Table 1 contains the locus revealed by probe Lam4-9.7.

Table 2 contains the restriction maps of Lam4-917 and of the genomic DNA segment of the locus. The 17.5 kilobase (kb) cloned human segment is represented by thick lines in both the Lam4-917 phage and the genomic representations. The letters B, E, and H indicate the sites for digestion with restriction endonucleases BamHI, EcoRI, and HindIII, respectively. Numbers below the phage diagram indicate the distance, in kb, from the left end of Lam4-917; numbers in the genomic diagram indicate the size, in kb, of fragments generated by HindIII

-10-

digestion and detected by Southern gel transfer hybridization analyses. The parentheses designate the polymorphic HindIII site revealed by probe Lam4-917.

Table 3 contains the linkage relationships of probe Lam4-917 to CF and probe Lam4-917 to PON. The maximum likelihood estimate is obtained for  $\theta$  at 0.14 with a lod (z) score of 3.96 (odds ratio: 9,100:1), if recombination frequencies are considered equal in male and female. The confidence interval for  $\theta$  is between 0.07 and 0.25 (19). The recombination fraction is close to the frequency of recombination detected by counts of the number of apparent recombinant chromosomes divided by the number of informative chromosomes among CF children (13/101).

Table 4 contains a family pedigree in a typical counseling situation. The darkened areas (1 and 4) represent CF alleles and the light areas (2 and 3) normal alleles.

Table 5 contains a family pedigree following diagnostic analysis using probe Lam4-917. The darkened areas represent CF alleles and the light areas normal alleles A, B, C, D are alleles of Lam4-917 generated by HindIII digestion and E, F, G, H are alleles of Lam4-917 generated by KincII digestion.

-11-

Table 6 contains a 2-point linkage map of chromosome 7 based on mapping with CEPH (Centre E'tude du Polymorphismes Humain) families where θ is recombination frequency between two markers and the LOD is as described below. Certain of the probes shown in this Table are further described in Table 7.

Table 7 contains a listing of flanking markers within 10 centimorgans of the CF gene. The Table contains the alleles revealed for each marker and their fragment links.

BRIEF DESCRIPTION OF PREFERRED EMBODIMENTS

The principle of genetic linkage is used to detect and follow autosomal recessive disease causing genes. The test does not require any knowledge of the biochemical basis of the disorder in order to test for its presence in an at risk individual or to identify a carrier of a mutant allele. The genetic location of such a disease gene is first determined by linkage studies with DNA RFLP probes and the probes that are linked are then used to study the inheritance of the disease gene in families known to be segregating the disease.

In cases where a single gene causes a disorder only one chromosomal location needs to be followed with genetically linked RFLP probes. In cases where more than one gene can give rise to the defect or phenotype, several chromosomal loci will necessarily be monitored unless it is possible to distinguish which gene is segregating in the particular family.

-12-

under study. The gene which is segregating may be deduced from the phenotype of the affected child in the family.

This invention defines the location of a cystic fibrosis gene by linkage studies using a RFLP DNA probe and a method of diagnosis for CF using DNA hybridization in gel transfer methods. It appears that the chromosomal location, i.e. CF locus, is responsible for most if not all of the CF cases included in the study herein. In addition, this test utilizes a simple technology and need only be performed once in the lifetime of an individual (who is at risk, putative carrier or normal). The information determined may have value for any future members of the family who may be at risk for cystic fibrosis.

Phenotypically neutral DNA sequence variations occur among individuals of any given species and arise from the insertion or deletion of DNA segments or, more commonly, by single nucleotide base changes. Some sequence differences change restriction endonuclease recognition sites or their spacing. The sequence difference is then revealed as variation in the length of restriction fragments recognized by a cloned DNA probe when the DNA of two or more individuals is examined. Preferably total genomic DNA is cleaved with a restriction enzyme,

separated according to size by electrophoresis,  
transferred and bound to a solid support such as a nylon  
membrane and hybridized to a radioactively labeled  
cloned DNA probe. The restriction fragment length  
5 polymorphism (RFLP) is visualized by autoradiography as  
a difference in the pattern of bands produced by  
hybridization of the probe to genomic DNA.

Family studies of ancestors of individuals at risk  
have shown that the variations in fragment lengths,  
10 RFLPs, are inherited in a Mendelian fashion with one  
form, allele coming from each parent. Since the RFLPs  
are stably inherited they can be used as genetic markers  
useful in the construction of a human genetic linkage  
map as proposed by Botstein, D., White, R.L.,  
15 Skolnick, M. and Davis, R.W. (1980) AM. J. Hum. Genet.

32, 314-331. The markers can be identified and located  
with RFLP probes which hybridize with the RFLP markers  
to reveal polymorphism at a single locus (e.g. a  
particular segment on a given chromosome). These same  
20 genetic markers can also be used to locate and follow  
the inheritance of certain traits including diseases  
such as cystic fibrosis. The closer the RFLP marker  
location is to the disease gene of interest the more  
likely it is to be co-inherited with the disease gene.  
For example, if a RFLP locus

-14-

is at a genetic distance of 10 centimorgans from a gene (recombination fraction of 10% or physical distance of approximately 10 million nucleotides), 90% of the time an RFLP allele from this locus will be coinherited with the disease locus since they are on the same segment of DNA but 10% of the time a crossover event will occur during meiosis such that a different RFLP allele from the locus will become attached to the DNA segment carrying the disease gene. At a genetic distance of 50 centimorgans from a gene, markers are said to be unlinked since a recombination event will occur approximately 50% of the time for each meiosis. In addition, it is very important that a large number of alleles at similar frequencies be present at a locus so that in any random mating there is a high probability that each parental chromosome contribution can be followed in the offspring. This measure of heterozygosity is referred to as the polymorphism information content or PIC (Botstein, et al. (1980) supra).

The present test utilizes cloned DNA probes that reveal polymorphism at a locus that is genetically linked to a cystic fibrosis locus. Therefore the inheritance of the disease cystic fibrosis can be detected by analysis of the RFLP alleles known to be cosegregating in families. Since the particular RFLP allele will vary from

-15-

family to family the particular allele co-inherited in an affected individual is the basis of the diagnosis in the family. Once this allele is identified it can be followed in all other individuals at risk that are descendants of the same two parents in the family. Thus the test is a linkage test that is preferably used as a family study rather than a population screening test.

In any linkage test there is the possibility that the two chromosomes that are being passed on to the child from the parents cross over (e.g. the mother's chromosomes break and rejoin with each other, exchanging genes). If there is only 1 marker in the test then the probability of a wrong diagnosis is simply related to the distance between the marker and the disease gene. Typically such a number might be 5% per parental chromosome resulting in a 10% error rate (i.e if the marker is 5 centimorgans (5% recombination) from the disease gene and since CF is a recessive, then, two disease genes are being passed on per generation). That is, 10% of the time a crossover will occur and it can't be determined that it has occurred, hence the 10% error rate.

With flanking markers one can tell when a crossover has occurred in the region. For example, if one marker is 5 centimorgans from the disease gene and a marker on the other side of the cystic

fibrosis gene is 3 centimorgans from the disease gene then, 16% of the time, a crossover event will occur in the transmission of the parental chromosomes to the child and the crossover will be detected (8% recombination for each parental chromosome or 16% total). The important difference here is that the accuracy of the flanking marker test is 99.7% versus 90% for the single marker test. The reason it is not 100% is due to cases in which two simultaneous crossovers between each of the markers and the disease gene (also known as a double crossover) that could occur, and this is calculated to be 0.3% of cases. So in our example 84% of the population (100% - 16% recombinants) will get a diagnosis that is 99.7% accurate (i.e. 0.3% error rate).

What can be done for the 16% of the population that has a documented crossover in an offspring at risk? These people can be given a partial diagnosis (in addition to knowing that a crossover has occurred) and in some cases this will help make a better risk assessment. One can note that there is a 5/8 probability that the crossover occurred in the interval between the marker, that is, 5 centimorgans from the disease gene and a 3/8 probability that the crossover occurred in the interval between the marker 3 centimorgans from the disease gene.

BAD ORIGINAL

-17-

Therefore, there is a probability of 38% that the child has CF and a 62% chance that it is a carrier. Depending on the marker distances from the disease gene these percentages will be of more or less usefulness in making a risk assessment. Of course the above illustration assumes that the markers are fully informative, that is, each parental chromosome can be distinguished at both marker locations.

The RFLP alleles are preferably detected by DNA hybridization with cloned preferably radioactively labeled DNA probes in gel transfer analysis of genomic DNA from family members, e.g. both parents, an affected child and the individual at risk.

The basis for this system of presymptomatic diagnosis for CF is the determination that a particular single-copy DNA probe called Lam4-917 defines a restriction fragment length polymorphism when used in gel-transfer hybridization methods and that the locus defined by this RFLP is linked to a gene that is responsible for a large fraction, if not all, inherited CF in humans and among caucasians. The linkage test can determine, to the precision indicated below, whether a child (even in utero) has inherited the defective (i.e. disease-causing) allele from one or both of its parents. If it inherited the disease allele only from one parent, it will be a "carrier" (not diseased but capable of passing on the disease to its offspring); if it inherited disease alleles from

-18-

both parents, it will have the disease; if from neither parent, then it will be free of CF.

The usefulness of the diagnostic test based on linkage is determined by three factors:

1. the degree of polymorphism as measured by the PIC;
2. the degree of linkage of the marker to the disease locus, measured by the recombination fraction observed between the RFLP locus and the disease gene, or, more conventionally by the genetic distance in centimorgans between them; or
3. the fraction of the disease caused by the particular disease gene: e.g. CF is caused by a single gene or, alternatively, some fraction of families with inherited CF may suffer because of another CF gene.

The more polymorphic an RFLP locus is, the more likely that a random individual will harbor different RFLP alleles than his or her spouse. In order for linkage to be followed, the RFLP probe which carries a radioactive or other marker capable of being detected, must be informative (i.e. heterozygous), and thus the PIC measures the usefulness of the probe in diagnosis. The probe Lam4-917 has a PIC of 0.58 (see Table 1), meaning that in its present form the probe will be useful in

-19-

more than 1/2 of all the families to which it is applied. The PIC of a particular probe can be improved by techniques familiar to one skilled in the art, and thus the present invention preferably utilizes Lam4 917 but improvements in its PIC or other probes and their improved PICs may be used.

Probes having PIC values of lower than 0.58 can be useful. However, when PIC values are below 0.58 it is preferred to use combinations of two or more probes in diagnosis in order to improve accuracy.

The degree of linkage (i.e. recombination fraction) between the locus defined by 917 and the major and possibly only CF locus is estimated to be about 0.15, meaning that 15% of the time the 917 allele in coupling with the CF allele will be exchanged so that the alternative allele of 917 is transmitted instead. Thus the linkage test using Lam4-917 in its present form can improve the odds of correct genotype to  $0.85 \times 0.85 = 0.7225$  for determination that a sibling of a CF child also has CF; the probability that a diagnosis of complete absence of the gene is in error is  $0.15 \times 0.15 = 0.0225$ , or 2.2. This is a distinct improvement over the current state of the art, which predicts odds of 0.25 (i.e. random) that a child has CF and 0.75 that it does not. Since the diseased gene has been located with the linked RFLP 917, the linkage can be improved by techniques familiar to one skilled in the art and other RFLP markers linked to the major CF gene as well as those markers located on the other side of the CF gene can be found using the

procedure described for 917. Techniques familiar to one skilled in the art to improve the linkage include "walking" along the chromosome using probe Lam4 917 screening libraries prepared from the chromosome on which the CF locus is located and preparing subsets of genomic libraries by using probe Lam4-917.

Linkage mapping efforts as those described above in relation to Lam4-917, have resulted in the linkage map of chromosome 7 shown in Table 6. Four of the markers are found to be within about 5 centimorgans of CF (designated L4-1033; S002, S158 and S094). These markers are very close to CF and to one another such that recombinants which might be useful for ordering markers with respect to each other and CF are rare making the precise ordering of the markers and their genetic distances from CF subject to some question. The map can be further resolved by such means as developing multilocus linkage analysis techniques and by analysing more family segregation data. Flanking markers as determined thus far and as shown in Table 6 include on one side of the CF locus :

S-140	S-148	S-241
S-199	S-201	S-003
S-124	L4-544	

-21-

and on the other side:

S-162, S-167, S-014, S-023, S-019, S-056, S-025,  
L4-917, S-146, L4-887, S-102, S-065, S-016, S-139,  
L4-751, S-155, L4-1020, S-060, S-244, S-166, S-161,  
S-194, L4-966, L4-281

Those markers listed above which are within 50 centimorgans of the CF locus are particularly desirable for use in the methods of this invention.

The above noted markers are all obtained from a chromosome 7 library obtained and treated as described in Example 1. The probes obtained are then analyzed and determined to have the linkage as noted in Table 6.

As used herein, the following terms have their known meaning in the art defined below:

**CHROMOSOMAL LOCUS.** A position on a chromosome defined either by means which allow physical localization or by linkage to other positions on the same chromosome.

**HETEROZYGOTE.** An individual possessing two forms, or alleles, of a polymorphic locus. One form is present on each chromosome of a homologous pair.

**LETHAL GENETIC DISEASE.** An inherited disorder which can lead to the death of the affected individual.

-22-

LINKAGE. A relationship between two chromosomal loci such that they are inherited together more than half the time. Linkage of two loci implies that they are located on the same chromosome.

MENDELIAN GENETICS. The study of traits determined by unique genes or sites. Named after Gregor Mendel (1822-84).

OBLIGATE HETEROZYGOTE. An individual who can be determined to be a heterozygote at particular chromosomal loci based on knowledge of the genetic constitution of other individuals in his/her family.

PHENOTYPE. The entire expressed physical, biochemical, and physiological constitution of an individual. When phenotype is used in the context of a single chromosomal locus, it refers to that portion of an individual's constitution which is affected by that locus.

PHENOTYPE. The entire expressed physical, biochemical, and physiological constitution of an individual. When phenotype is used in the context of a single chromosomal locus, it refers to that portion of an individual's constitution which is affected by that locus.

PHENOTYPICALLY NEUTRAL. Having no effect on phenotype.

POLYMORPHISM (or genetic heterogeneity). Alternate forms observed at a chromosomal locus.

-23-

SEQUENCE POLYMORPHISM. A polymorphism resulting from differences in the DNA or protein sequence observed in different individuals at the same chromosomal locus.

It has been estimated that approximately 70% of the human genome is comprised of unique i.e. single-copy DNA sequence and 20% mid-repeat sequences (approximate repeat 2-1000 times per genome) while about 10% is highly repeated 50- $10^5$  fold repeat sequences (Strayer et. al., 1985). While polymorphism no doubt exists in all three classes of human sequence, variation between individuals at single-copy sequence regions is most useful for the genetic testing described above. Only unique sequence regions are tested for RFLPs by hybridization of cloned single-copy DNA sequences to restriction endonuclease cleaved DNAs from unrelated individuals in gel-transfer experiments (Wyman, A. and White, R. (1980) Proc. Natl. Acad. Sci. USA 77, 6754-6758; and White et al. 1985).

The probes are preferably derived from whole or partial human genomic libraries which have been constructed and contain cloned DNA segments 10-20 kilobases in length (Lawn, R. W., Fritsch, E. F., Parker, R. C., Blake, G. and Maniatis, T. (1978) Cell 15, 1 157-1174). Putative single copy clones are identified preferably from plaque filter

- 24 -

hybridization experiments in which total genomic human DNA is labeled with  $^{32}\text{P}$  via a nick translation reaction (Rigby, P. W. J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237-254.) and hybridized to filter replicas of bacteriophage clones containing human DNA inserts plated on a lawn of E. coli (Wyman et al. (1980) supra). Bacteriophage plaques that show hybridization signals contain middle repetitive to highly repetitive human DNA sequences; these are avoided in favor of those that contain human DNA but do not hybridize to the total human DNA probe.

Each candidate single copy human DNA clone can be tested for the ability to reveal polymorphism by hybridization to restriction enzyme cleaved genomic DNA from random unrelated individuals. The bacteriophage clones containing the human DNA inserts are radioactively labeled with  $^{32}\text{P}$  via nick translation and hybridized in gel transfer experiments to genomic DNAs. A standard screening blot consisting of 5 unrelated individuals cleaved with each of 6 restriction enzymes (e.g. MspI, TaqI, HindIII, ECORI, BglII, BamHI) is efficient in detecting polymorphism. Since 10 chromosomes are scored, a two allele polymorphism (frequency of each allele 50%) or greater than 2 allele polymorphism will be detected, if present, with a certainty of greater than 90% using such a screening panel.

-25-

Once polymorphism has been detected follow up gel transfer experiments can be done with panels of DNA (cleaved with the appropriate restriction enzyme) from more random unrelated individuals (e.g. 20) so that an estimate of the frequency of the RFLP alleles can be made and PIC can be estimated. In addition, other enzymes can be tested to see if additional variation can be detected. It is important to confirm that the polymorphism represents a single locus. This is done by studying the inheritance of the RFLP alleles in families. The presence of more than one locus would be suspected if non-Mendelian segregation of the banding pattern in the autoradiograms is observed. In some cases the polymorphic patterns are very complicated and alleles can only be defined from such inheritance studies. Useful family pedigrees for these analyses preferably contain the 4 grandparents, the parents, and large sibships (6 or more children) from which DNA can be purified from blood samples obtained or isolated from immortalized lymphoblastoid cell lines (Camden Cell Repository; White et. al., 1985).

Example 1:

In a first example of this invention the isolation and identification of Lam4-917 is described.

-26-

The isolation and characterization of Lam4-917 begins with the HaeIII-AluI partial digest library in the lambda vector Charon 4A (Lawn, et al. (1978) supra). The method used for isolating Lam4-917 is according to Wyman, et al (1980) supra and Maniatis, T., Hardison, R.C., Lacy, E., Laver, S., O'Connell, C., Quon, D., Sim, G.K., Efstradiatis, A (1978) Cell 15, 687-701.

Plaque hybridization of genomic DNAs is carried out by incubating approximately 150 bacteriophage of the HaeIII-AluI library in an equal volume of SM (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 8 mM MgSO<sub>4</sub>) containing E. Coli strain CGE6 (CGE6 = HB101: F-, hsdS20(r-B<sup>m</sup>-B), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm<sup>r</sup>), xyl-5, mtl-1, supE44, lambda- as described by Bolivar, F. and Backman, K. (1979) Methods Enzymol. 68, 645, and Boyer, H. W. and Roulland-Dussoix, D. (1969) J. Mol. Biol. 41, 459) in a total volume of 2.5 ml. The mixture is incubated at 37°C for 15 minutes and then 3 ml LB top agarose is added, mixed and poured onto an LB (Luria Broth per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar) plate. The plate is incubated at 37°C overnight. Following incubation, the plates are chilled at 4° C for one hour. Two plaque lifts per plate are done for one minute and two minutes. The plaque lifts on a filter are

-27-

denatured for one minute, neutralized for five minutes, rinsed in 2X SSC (1X = 0.15 M NaCl, 0.015 M NaCitrate) and air dried for about two hours. The filters from both plaque lifts are baked at 80°C in a vacuum oven for two hours and then stored at room temperature.

Nick translation of the human probe is according to Rigby, et al. (1977) supra. Nicking the DNA is carried out for ten minutes at 13° C followed by five minutes at 65° C in a final volume of 8  $\mu$ l containing DNase I cocktail (50 uUnits DNase I, 50 mM Tris, pH 7.5, 5 mM Mg Cl<sub>2</sub>, 20 mM  $\beta$ -mercaptoethanol, 0.05% gelatin), 0.25  $\mu$ g human genomic DNA and 5 ng plasmid (pCGE40 contains about 10,000 copies of lac Z). The 8  $\mu$ l nicked DNA (0.25  $\mu$ g) was incubated at 13° C for 70 minutes with 7  $\mu$ l Pol I cocktail (Pol I cocktail contains 50  $\mu$ l <sup>32</sup>P-dCTP from New England Nuclear, 10  $\mu$ l 0.3 mM each dATP, dGTP and dTTP, 5  $\mu$ l DNA polymerase I (50 units) from New England Biolabs and 5  $\mu$ l distilled water for a final total volume of 70  $\mu$ l. The reaction is terminated by the addition of 35  $\mu$ l stop mix (stop mix contains 100  $\mu$ l 0.5 M EDTA, 10  $\mu$ l 10 mg/ml tRNA and 240  $\mu$ l distilled water). The reaction mixture is loaded onto a 0.8 cm x 3.5 cm Biogel P-60 column preequilibrated with TE buffer (10 mM Tris, 10 mM Na<sub>2</sub>EDTA at pH 8.0 and washed

-28-

with same. The location of the  $^{32}\text{P}$ -labeled human DNA probe is followed by counting samples of each fraction in a scintillation counter and the first peak is collected.

The labeled human DNA probe is hybridized to the blotted genomic DNA according to the following procedure. The first set of filters from the stored plaque lift filters are floated on 6X SSC and then submerged for about 10 minutes. The filters are prewashed in 660 ml prewash solution (50 mM Tris-HCl, pH 8.0, 1.0 M NaCl, 1.0 mM EDTA, 0.1% SDS) for about 2 hours in a 42° C shaker and then prehybridized in 150 ml prehybridization solution (5X SSC, 0.40 M sodium phosphate, pH 6.5, 0.1 mg/ml salmon sperm DNA, 10% dextran sulfate, 50% formamide, 5X Denhardt's Ficoll 400, polyvinylpyrrolidone 360, bovine serum albumin) for about 2.5 hours in a 42° C shaker. Following prehybridization, 200  $\mu\text{l}$  of a solution containing greater than  $10^8$  dpm human DNA probe is added and incubation in the 42° C shaker is continued overnight. Hybridization with the nick-translated probe is by the methods of Southern, E. M. (1975) J. Mol. Biol. 98, 503-517, and Barker, D. et al (1984) Am. J. Human Genetics 36 1159-1171 and are carried out for about 22 hours. Following hybridization, filters are washed for 30 minutes at room

-29-

temperature in a solution containing 2X SSC and 0.1% sodium dodecylsulfate (SDS) and then 60 minutes at 65° C in 0.5X SSC and 0.5% SDS. Autoradiography was for 17 hours at -70° C with Kodak XAR-5 film, backed by a Lightening-Plus intensifying screen (DuPont).

The genomic DNA plaques undergo a second screening. This second screening is carried out as described above for plaque hybridization except that bacteria phage are applied to a lawn of strain CGE6 with a toothpick, nick translation of the human probe and hybridization of the labeled probe to the blotted DNA. Following two rounds of screening, Lam4-917 is found to hybridize to the genomic DNA, is a clear plaque and, therefore, a putative single copy DNA clone. A phage plate lysate is prepared as is well known in the art (Arber, W., Enquist, L., Hohn, B., Murray, N. and Murray, K. (1983) *Exp. Methods for Use with Lambda*, Lambda II, ed. F. Hendricks, J. Roberts, F. Stahl, R. Weisberg, 433-466 Cold Spring Harbor) from the clear plaque identified as Lam4-917. To obtain the phage lysate, approximately  $10^5$  phage particles are incubated with 100  $\mu$ l CGE6 overnight at 37° C for 15 minutes. LB top agarose (3 ml) is added to the incubated solution and then the total solution is poured onto LB plates. The plate is incubated at 37° C for 8 hours and then 3.5 SM is added to the plate and the

-30-

plate is incubated at 4° C overnight. The phage titer is about  $1-2 \times 10^{10}$  pfu/ml. The SM is pipetted from the phage lysate, a few drops of chloroform is added and the solution is vortexed for about 10 seconds. The lysate is spun in two microcentrifuge tubes for 15 minutes at 4° C, and then transferred to a screw-cap tube and stored at 4° C.

A DNA miniprep is prepared from the phage according to Davis, R. W., Botstein, D. and Roth, J. R. (1980) "A Manual for Genetic Engineering. Advanced Bacterial Genetics", Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., 109-111 and left in isopropanol at -20° C overnight. The phage DNA is collected following centrifugation in a microcentrifuge for 15 minutes at 4° C. The DNA pellet is air-dried and resuspended in  $20\mu\text{l}$  TE, pH8.0. A 100 ng sample of the phage DNA ( $1\mu\text{l}$  DNA plus  $9\mu\text{l}$  stop mix) is run on a 1% agarose gel in TA buffer at 60 volts (about 90 mAmps) together with a lambda DNA control to determine that the human DNA clone is isolated and results confirm that the clone of interest is isolated.

Lam4-917 is nick translated using conditions described previously and hybridized to restriction enzyme cleaved genomic DNA. The restriction enzyme cleaved genomic DNA blot consists of five unrelated

-31-

individual DNA restriction cleaved with each of six restriction enzymes: MspI, TaqI, HindIII, EcoRI, BglII and BamHI. The genomic DNA are restricted separately with the appropriate restriction enzyme according to the following procedure. Enough of the DNA solution for 100ug (usually 400ul) is added to a 1.5 ml Eppendorf tube together with the appropriate amount of the specific endonuclease digestion buffer made to the manufacturer's recommendations, restriction endonuclease in a 2 to 5 fold excess, 2 mM spermidine-HCl and distilled water, if necessary, to adjust to the final reaction volume of 600  $\mu$ l. The mixtures are incubated at 37° C overnight with the exception of the TaqI digests which are incubated at 65° C. The human DNA is monitored for complete digestion by digesting lambda phage DNA in the presence of a sample of the human digest. Digestion of the human DNA is judged to be complete if the lambda DNA is fully digested. The reaction is terminated with stop reactants (final concentration - 10 mM EDTA, pH 7.0, 0.5% SDS and 0.3 M NaCl) and stored for future electrophoresis. Prior to electrophoresis, marker dye is added to each sample and then loaded on the gel. The DNAs are subjected to electrophoresis on 0.8% agarose gels submerged in a Tris-acetate EDTA buffer. Following electrophoresis the DNA is denatured in th

-32-

gel with a solution of 0.2 N NaOH, 0.6 M NaCl, neutralized with 0.5 M Tris-HCl pH 8.0, 1.5 M NaCl and transferred overnight (16 hours) to a nylon membrane (zeta-bind, AMF Cuno) in 25 mM sodium phosphate pH 6.5. The nylon membrane is washed then baked for 2 hours in a vacuum oven at 80° C. The DNA containing nylon membrane is then prehybridized 2 hours at 42° C in a solution containing 5x SSC 40 mM sodium phosphate, pH 6.5, 0.1 mg/ml salmon sperm DNA, 10% dextran sulfate, 50% formamide, 5x Denhardts Ficoll 400, polyvinylpyrrolidone 360, bovine serum albumin. The RFLP 917 DNA probe is labeled with  $^{32}\text{P}$  to a specific activity of greater than  $10^8$  dpm/g via a nick translation reaction with DNA polymerase I (New England Biolabs) and alpha- $^{32}\text{P}$  NTPs (New England Nuclear) as described previously. Labeled DNA is separated from unincorporated nucleotides by chromatography on Biogel P-60 (Bio-Rad). The  $^{32}\text{P}$  labeled DNA is denatured by boiling for 5 minutes then added to the prehybridization solution containing the nylon membrane. Hybridization is carried out at 42° C for 20 hrs. Following hybridization, filters are washed as described previously. Autoradiography is done for 1-5 days at -70° with Kodak XAR-5 film and intensifying screens (Dupont Cronex Lightening Plus).

-33-

The results of the Southern hybridization show that Lam4-917 can detect a HindIII two allele polymorphism. The HindIII polymorphism is verified by rescreening Lam4-917 on a HindIII panel of human genomic DNA as described previously. Results are consistent with the previous finding that Lam4-917 reveals a HindIII two allele polymorphism. In addition, a HincII polymorphism is revealed on the former panel which revealed the HindIII polymorphism. The Lam4-917 marker is rescreened on a HincII genomic DNA panel and Southern hybridization results verify that Lam4-917 can detect both a HindIII two allele polymorphism and a HincII polymorphism.

The restriction map of Lam4-917 is determined by the cos-oligonucleotide method according to Rackwitz, H., Zehetner, G., Frischauf, A. and Lehrach, H. (1984) Gene 30, 195-200 and by standard restriction analysis of subcloned EcoRI fragments (Table 2). The correspondence of the restriction sites in the clone, which contains 18 Kb (kilobases) of human DNA, with those in the human genome is confirmed by Southern gel-transfer hybridization analysis as described previously.

Linkage of Lam4-917 to the major CF locus is made by application (hybridization) of the 917 RFLP probe to 39 nuclear families containing two or more

-34-

offspring with positively diagnosed CF. The data is analyzed by the likelihood method of linkage analysis via a standard computer program (LIPED 3.3) available to one skilled in the art of human genetics and according to Ott, J. (1974) Amer. J. Human Gen. 26, 588-597 and Ott, J. (1985) "Analysis of Human Genetic Linkage", in The Johns Hopkins Univ. Press, Baltimore, Md., 22-119 (Table 3). The LOD score is a measure of the relative likelihood that there is linkage (at a given recombination fraction) and the alternative hypothesis that the data arose by chance and no linkage exists. The value generally accepted as conclusive is a LOD score of 3. The LOD score for Lam4-917 is 3.96 at a recombination fraction of 0.14. Formally, such a score means that the odds are about 10,000 to 1 that there is linkage at this distance as opposed to non-linkage. In order to determine if there is linkage between CF and PON, paraoxonase typing data is analyzed from the CF families. Eleven of the 31 families with available data are informative for this analysis. A tight linkage is detected between Lam4-917 and PON (Table 4). A maximal likelihood estimate for  $O$  at 0.05 is obtained with a LOD score of 5.01 (odds ratio: 102,000:1) and the confidence interval between 0.01 and 0.17. A maximal LOD score of 5.13 (odds ratio: 132,000:1) is obtained at  $O_M$  of 0.10 and  $O_F$  of 0.04.

-35-

The probe Lam4-917 was hybridized to DNA from a set of rodent-human hybrid cell lines containing various subsets of the human chromosome complement (Shows, T.B., Sakaguchi, A.Y., Naylor, S.L. (1982) Adv. Hum. Genet. 12, 341-452). Results from 25 cell lines tested indicate that the clone Lam4-917 is derived from human chromosome 7. Hybridization results are concordant with the presence or absence of chromosome 7 in 22 lines, and discordant in only one.

Assignment of Lam4-917 to chromosome 7 was testing additional somatic cell hybrid panels lines and by in situ hybridization to metaphase chromosomes.

Example 2:

This example illustrates diagnosis of cystic fibrosis in counseling situation.

A typical counseling situation appropriate for application of the CF RPLPS test of this invention is one shown by the family pedigree in Table 4; a CF child is born thus indicating to the parents that they each carry a CF allele. The parents wish to continue having children and want to know the status of a living fetus at risk carried by the mother, i.e. is it affected with cystic fibrosis, is it a carrier or is it free of either disease gene.

-36-

The CF allele at the locus revealed by the Lam4-917 probe is responsible for CF in this family and accordingly the alleles revealed by Lam4-917 can be used to follow CF in this family.

Blood samples (1-20 ml) are collected from the mother, father and affected child and the DNA is purified according to the following procedure. Cells are first lysed by addition of a ten fold volume of buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.6, 5 mM MgCl<sub>2</sub>, 1% triton - X-100) and nuclei are isolated by centrifugation. The nuclear pellet is then suspended in a small volume of 75 mM NaCl, 25 mM EDTA, 1% SDS and incubated with proteinase K (10mg/ml) overnight (12-16 hrs.) at 42°C. Following a 2-4 hour extraction at room temperature of the solution with Tris-EDTA saturated phenol, the aqueous solution is re-extracted with a solution of chloroform and isoamylalcohol (24:1) and the DNA is precipitated by addition of one tenth volume of 3 M NaCl and 1.5 volume isopropanol. The DNA is dissolved in 0.1 x TE buffer and reprecipitated by addition of 1/2 volume 7.5 M ammonium acetate and 2 volumes of cold 95% ethanol. The precipitated DNA is resuspended in 10 mM Tris-HCl, pH 7.5, 1mM EDTA to a concentration of 250 ug/ml and stored at 4° C. The DNA yield is approximately 100-200 ug per 10 ml peripheral blood of 10 ug per 10<sup>6</sup> cells.

-37-

DNA is obtained from the fetus at risk as from the cells obtained from an amniotic fluid sample. The DNA is purified as described above.

The four DNAs (father, mother, affected child and fetus at risk) are each digested with restriction enzymes HindIII and HincII separately. DNA samples at a final concentration of 175  $\mu$ g/ml are digested to completion (4-6 hours) with restriction endonucleases HindIII and HincII (5 units restriction enzyme/ $\mu$ g DNA) under conditions recommended by the supplier (New England Biolabs). In order to check the completeness of the digestion aliquots from the reaction mixture containing 1  $\mu$ g human genomic DNA are added to an identical reaction mixture containing 1 ug bacteriophage lambda DNA. A limit digest of the genomic DNA is assumed if the digest pattern of lambda DNA showed complete cleavage as measured by ethidium bromide staining of the DNA size-fractionated by electrophoresis in a 0.8% agarose gel (Sigma). Fragments from the four restricted DNAs are separated according to size by electrophoresis through a 0.8% agarose gel and transferred to a solid support nylon membrane as described in Example 1. DNA probe Lam4-917 is radiolabeled with  $^{32}$ P and hybridized to the enzyme cleaved DNAs from the 4 family members. Results are interpreted from autoradiographs of the hybridized membrane.

-38-

As shown in Table 5, since the affected child carries both mutant alleles at the CF locus, the RFLP alleles revealed at the 917 locus are those associated with the mutant CF allele itself thus alleles B and D (HindIII digest) and F and H (HincII pattern) correspond to the mutant alleles.

Therefore these alleles are the mutant alleles carried by each parent whereas the alleles ACEG are linked to normal CF genes in the parents. Analysis of the hybridization pattern of the fetus at risk shows that alleles ACEG are inherited from the parents indicating that the child has a normal genotype and will not develop CF or pass on a mutant gene to any offspring except that a 15% recombination factor has to be considered for each allele of 917 because of the genetic distance between the CF locus and the 917 locus. Therefore the diagnosis must be interpreted with this probability factor included.

**Example 3:**

This example illustrates diagnosis of cystic fibrosis using flanking markers.

A CF child is born thus indicating to the parents that they each carry a CF allele. The parents wish to continue having children and want to

BAD ORIGINAL

-39-

know the status of a living fetus at risk carried by the mother, i.e., is it affected with cystic fibrosis, is it a carrier or is it free of the disease gene.

The CF allele at the locus is revealed by two flanking markers (Lam4-917 and X where X is S-094, S-002, L4-1033 or S-158). For example illustration, the markers are Lam4-917 and S-002 and they are fully informative in this family and accordingly the alleles revealed by them can be used to follow CF in this family. DNA is obtained, digested with restriction enzymes (HindIII and IncII for Lam4-917 and TaqI for S-002) and probed with the markers as described in Example 2.

Analysis of the hybridization patterns of the fetus at risk shows inherited alleles from the parents are normal alleles indicating that the child has a normal genotype and will not develop CF or pass on a mutant gene to any subsequent offspring. Since in Example 2 only one marker was being used, a 15% recombination factor had to be considered for each allele of Lam4-917 because of the genetic distance between the CF locus and the locus as defined by Lam4-917 and the probability that the diagnosis is correct using this one marker is about 70%. In the present Example using flanking markers and where no crossover has occurred, there is a 15%

-40-

recombination factor between S-002 and Lam4-917 and a 5% recombination factor between S-002 and CF making the percent error of 0.4% or a probability of accuracy of 99.6% in this case.

While specific embodiments of the invention have been shown and described above, many variations are possible. The DNA used for testing can be obtained from blood samples, amniotic fluid, chorion tissue samples or from other biofluids and cells where DNA is present.

The DNA can be purified from the samples taken in any known manner. Normally the cells are treated to rupture the cell walls and release the contents whereupon foreign matter and proteins are removed from the DNA so as to purify it before testing. Conventional centrifugation, extractions and the like can be used.

The restriction digesting procedure can use standard techniques known in the art. While HindIII and HincII restriction enzymes are used with Lam4-917, other restriction enzymes can be used when other CF locus identifying probes are used as known in the art.

The electrophoresis step can use parameters known in the art for separating restriction digested DNA segments.

BAD ORIGINAL

-41-

The solid support to which the electrophoretically separated DNA is transferred can be a nylon membrane, paper or other solid supports as known in the art. In some cases, the support need not be used if good separation of digested DNA segments can be obtained in a readable form using standard techniques.

Hybridization techniques known in the art can vary greatly. The double stranded DNA to be tested is disassociated as with suitable chemicals, temperature or varied ion concentrations. The single copy probe is then applied to single stranded DNA, under conditions which permit rehybridization.

In some cases the use of restriction enzymes can be carried out simultaneously with deposit and/or separation of the DNA in a suitable support.

While the invention specifically describes use of a single probe in each test as in Example 2, plural probes can be used in each test in the testing procedure as in Example 3. Such multiple probes can be flanking markers or multiple linked probes.

We hereby incorporate by reference all of the publications recited herein and also the attached paper entitled "Cystic Fibrosis Locus Defined by a Genetically Linked Polymorphic DNA Marker" by Le;e-Chee Tsui et al pages 0-21.

BAD ORIGINAL

-42-

CLAIMS

1. A method of determining presence or absence of one or more cystic fibrosis alleles in a DNA sample taken from an individual having a family genetic background in which family DNA of ancestors in the family line contains identified cystic fibrosis causing allele genes, said method comprising,

restriction digesting a DNA sample to be tested, which DNA sample is from an individual having a family genetic background in which family ancestors of the family have DNA which contains identified cystic fibrosis allele;

applying a probe to said DNA sample which probe hybridizes within less than 50 centimorgans of a cystic fibrosis gene in said family DNA,

and determining whether or not hybridization has taken place in a manner consistent with the presence of one or more cystic fibrosis alleles in said sample.

2. A method in accordance with the method of claim 1 wherein said DNA is first purified by rupturing cell walls and obtaining said DNA sample to be tested in purified form prior to restriction digesting.

-43-

3. A method in accordance with claim 2 and further comprising separating said restriction digested DNA by electrophoresis prior to applying said probe.

4. A method in accordance with the method of claim 3 and further comprising transferring said electrophoretically separated DNA to a solid support prior to applying said probe.

5. A method in accordance with the method of claim 1 wherein said probe hybridizes within 15 centimorgans of said cystic fibrosis gene and said digesting is carried out with Hind III and Hinc II.

6. A method in accordance with the method of claim 5 wherein said probe carries a marker for identifying DNA band positions which exhibit hybridization on said solid support.

-44-

7. A method in accordance with the method of claim 4 wherein said determining is carried out by comparing the results of hybridization with the results obtained from DNA of the parents and a sibling exhibiting the disease of the individual from whom the sample to be tested was taken, so that hybridization at similar positions on the support with respect to each parent and a sibling exhibiting the disease indicates the presence or absence of a cystic fibrosis allele obtained from each parent in the DNA tested.

8. A method in accordance with the method of claim 5 wherein said determining is carried out by comparing the results of hybridization with the results obtained from DNA of the parents and the sibling exhibiting the disease of the individual from whom the sample to be tested was taken, so that hybridization at similar positions on the support with respect to each parent and sibling indicates the presence or absence of a cystic fibrosis allele obtained from each parent in the DNA tested.

9. A method in accordance with claim 1 wherein said probe is Lam4-917.

10. A method in accordance with claim 7 wherein said probe is Lam4-917.

-45-

11. A method in accordance with claim 4 wherein said probe is Lam4-917.

12. In a method of identifying whether or not an allele of a cystic fibrosis gene is present in a DNA sample taken from an individual, the improvement comprising applying a probe under hybridizing conditions to said DNA sample which probe identifies a cystic fibrosis locus specific to a cystic fibrosis gene in said DNA if present and determining whether or not said probe has hybridized to said DNA.

13. A method in accordance with the method of claim 12 wherein said probe hybridizes within less than 50 centimorgans of a cystic fibrosis gene locus.

14. A method in accordance with the method of claim 12 wherein said probe hybridizes within 15 centimorgans of a cystic fibrosis gene locus.

15. A method in accordance with the method of claim 12 wherein said probe has a PIC value of at least 0.58.

16. A method in accordance with the method of claim 12 wherein said probe is Lam4-917.

BAD ORIGINAL

-46-

17. A probe which hybridizes within less than 50 centimorgans of a cystic fibrosis gene locus.

18. A probe which hybridizes within 15 centimorgans of a cystic fibrosis gene locus.

19. Lam4-917.

20. In a method of determining the presence or absence of a cystic fibrosis gene, the use of a DNA probe obtained from a gene library of human genomic DNA, said probe having a PIC value of at least 0.58.

21. A method in accordance with the method of claim 20 wherein said probe is applied to DNA to be tested after digesting said DNA to be tested with Hind III and Eco RI.

22. A method of determining presence or absence of one or more autosomal recessive trait alleles in a DNA sample taken from an individual having a family genetic background in which family, DNA of ancestors in the family line contains said trait, said method comprising, obtaining a DNA sample from said individual,

restriction digesting said DNA sample to be tested,

-47-

applying a probe to said DNA sample which hybridizes within less than 50 centimorgans of a gene for said autosomal recessive trait, in said family DNA,

and determining whether or not hybridization has taken place in a manner consistent with the presence of one or more said alleles in said sample.

23. In a method of identifying whether or not an autosomal recessive trait gene is present in a DNA sample taken from an individual, the improvement comprising applying a probe under hybridizing conditions to said DNA sample which probe identifies an autosomal recessive trait locus specific to an autosomal recessive trait gene in said DNA if present and determining whether or not said probe has hybridized to said DNA.

24. A cystic fibrosis gene locus which is within 15 centimorgans of a portion of attached DNA which portion hybridized to Lam4-917.

25. A method in accordance with the method of Claim 1 and further comprising applying at least one additional probe to said restriction digested DNA sample to be tested.

-48-

26. A method in accordance with the method of Claim 25 wherein said at least one additional probe is a flanking marker to said first mentioned probe.

27. The improvement of Claim 20 wherein a second DNA probe obtained from a gene library of human genomic DNA having a pick value of at least 0.58 is used in conjunction with said first mentioned probe and is a flanking marker to said first mentioned probe.

29. A method in accordance with the method of Claim 22 and further comprising applying a second probe to said DNA sample after said restriction digesting, which second probe hybridizes within less than 50 centimorgans of a gene for said autosomal recessive trait in said family DNA and which second probe is a flanking marker to said first mentioned probe.

30. A cystic fibrosis gene locus which is within 15 centimorgans of a portion of attached DNA which acts as a marker hybridized thereto on one side of said locus and a second portion of attached DNA which second portion hybridizes on a second side of said gene locus.

Table I..

0226288

Probe - Lam4-917

Enz - HindIII

PIC - 0.36

# Chromosomes examined - 32

Alleles/ Fragment lengths - (1) 6.2 Kb  
(2) 5.1, 1.0

Allele Frequency - (1) 0.625  
(2) 0.375

Const. Fragments (Kb) - 12.5, 7.6

related Pm - none

Unrelated Pm - HincII

---

Probe - Lam4-917

Enz - HincII

PIC - 0.35

# chromosomes examined - 32

Alleles/ Frag lengths (Kb) - (1) 4.3  
(2) 2.3, 1.8

Allele Frequencies - (1) 0.67  
(2) 0.34

Const frags (Kb) - 6.8, 5.1, 3.9

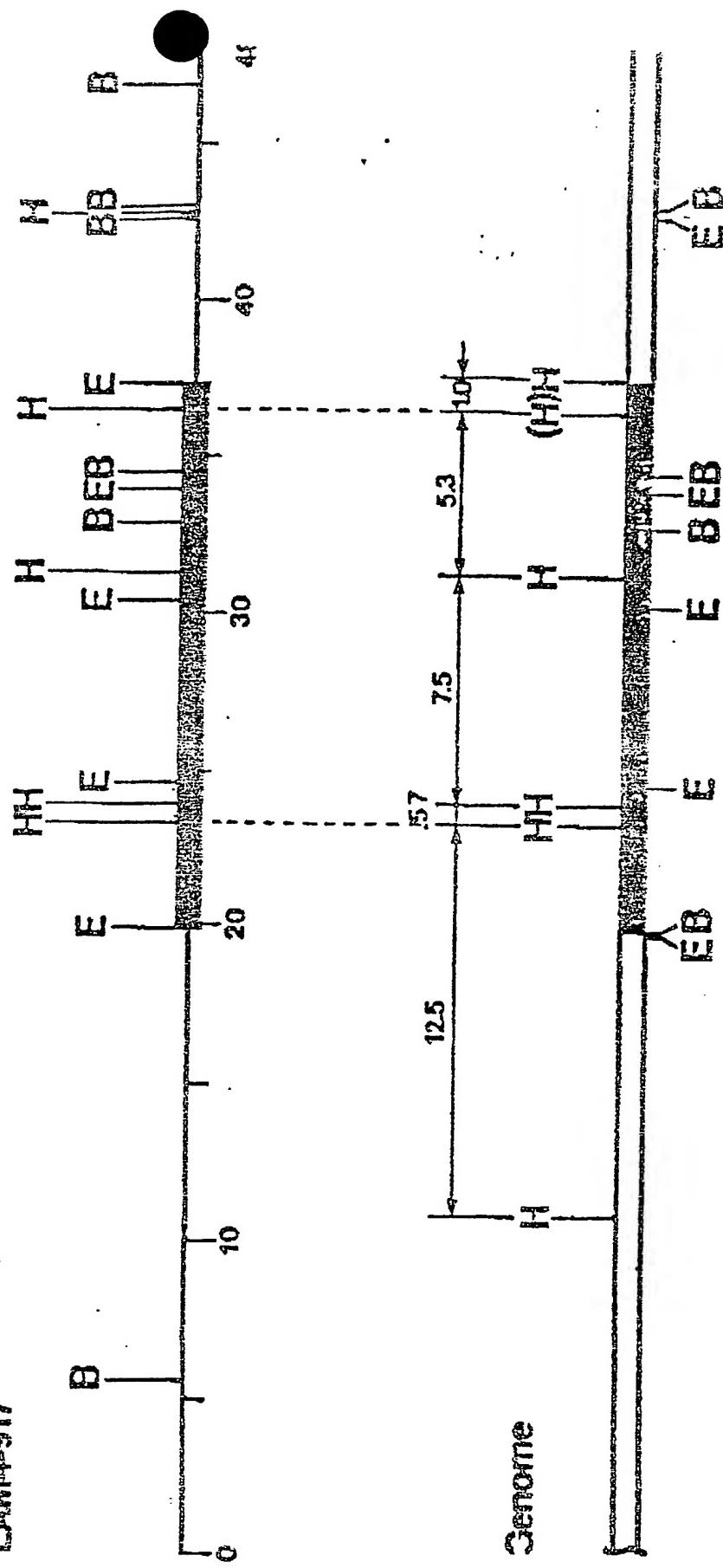
Related Pm - none

Unrelated Pm - HindIII

---

Combined PIC HindIII + HincII = 0.38

LAMA-917



0226288

TABLE 2

TABLE 3

## Linkage Relationships

Locl	Number of informative families	LOD (z) scores at recombinant fractions ( $\theta$ ) of:								
		0.01	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40
Lam 4-817-CF	36 (Can)	-6.02	0.98	2.84	3.20	2.96	2.44	1.79	1.13	0.55
	3 (HGCMR)	0.14	0.69	0.79	0.75	0.66	0.53	0.39	0.25	0.12
	Total	-5.88	1.67	3.63	3.95	3.62	2.97	2.19	1.38	0.67
		[ $\hat{\theta} = 0.14$ (z = 3.96); confidence interval: 0.07–0.25; $\hat{z} = 4.13$ ( $\theta_M = 0.09$ ; $\theta_F = 0.19$ )]								
Lam 4-817-PON	11 (Can)	4.27	5.01	4.78	4.26	3.66	2.87	2.25	1.51	0.81
		[ $\hat{\theta} = 0.05$ (z = 5.01); confidence interval: 0.01–0.17; $\hat{z} = 5.12$ ( $\theta_M = 0.10$ ; $\theta_F = 0.04$ )]								

0226288

4/7

Mode of Inheritance Recessive

Risk Summary:

Fully Informative RFLP Marker,  $\theta = 0.15$

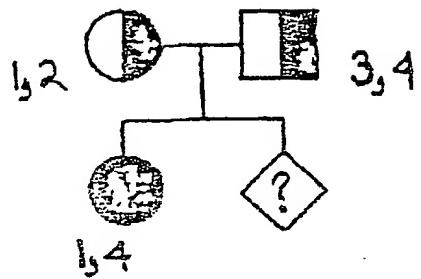


TABLE 4

022628

TABLE 5

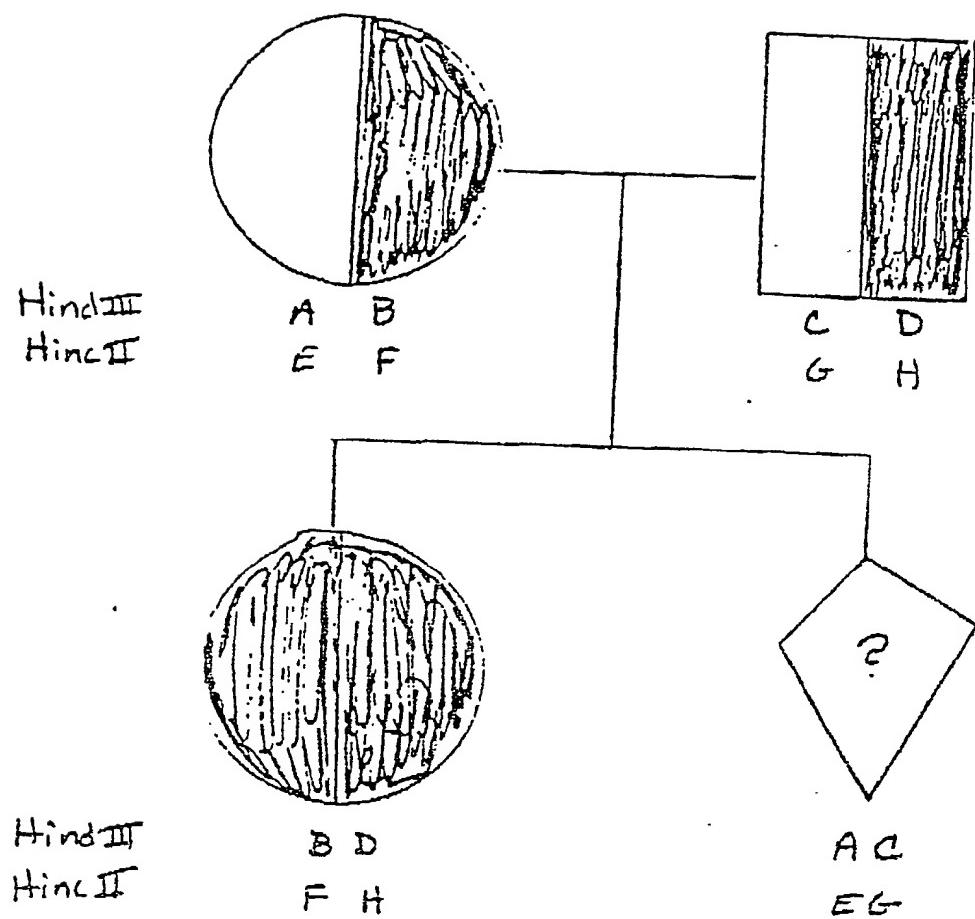


TABLE 6

Probe	Enzyme	Variable Fragment	Constant
		Length (Kb)	Fragment Length (Kb)
5	S-140	Allele 1 - 5.4 Kb	1.7 Kb
		Allele 2 - 5.7 Kb	
10	S-002	Allele 1 - 4.3 Kb, 2.4 Kb	1.0 Kb
		Allele 2 - 2.9 Kb, 2.0 Kb	
15	S-094	Allele 1 - 19.0 Kb	none
		Allele 2 - 14.0 Kb, 7.8 Kb	
20	S-158	Allele 1 - 6.5 Kb	3.3 Kb, 2.2 Kb
		Allele 2 - 5.3 Kb	
25	L4-1033	Allele 1 - 12.4 Kb	8.8 Kb
		Allele 2 - 10.5 Kb	
30	S-162	Allele 1 - 10.9 Kb	2.3 Kb
		Allele 2 - 10.4 Kb	
35	S-157	Allele 1 - 9.8 Kb, 3.8 Kb	none
		Allele 2 - 2.7 Kb, 1.7 Kb, 1.2 Kb	
40	S-014	Allele 1 - 18.0 Kb	8.0 Kb
		Allele 2 - 15.0 Kb	
45	S-023	Allele 3 - 14.5 Kb	
		Allele 1 - 10.5 Kb	1.0 Kb
		Allele 2 - 3.4 Kb	

Table I.

0226288

Probe - Lnm4-917

Enz - BindIII

PIC - 0.36

# Chromosomes examined - 32

Alleles/ Fragment lengths - (1) 6.2 Kb  
(2) 5.1, 1.0

Allele Frequency - (1) 0.625  
(2) 0.375

Const. Fragments (Kb) - 12.5, 7.6

Related Pm - none

Unrelated Pm - HincII

---

Probe - Lnm4-917

Enz - HincII

PIC - 0.35

# chromosomes examined - 32

Alleles/ Frag lengths (Kb) - (1) 4.3, 1.9  
(2) 2.3, 1.9

Allele Frequencies - (1) 0.67  
(2) 0.33

Const frags (Kb) - 6.0, 5.1, 3.9

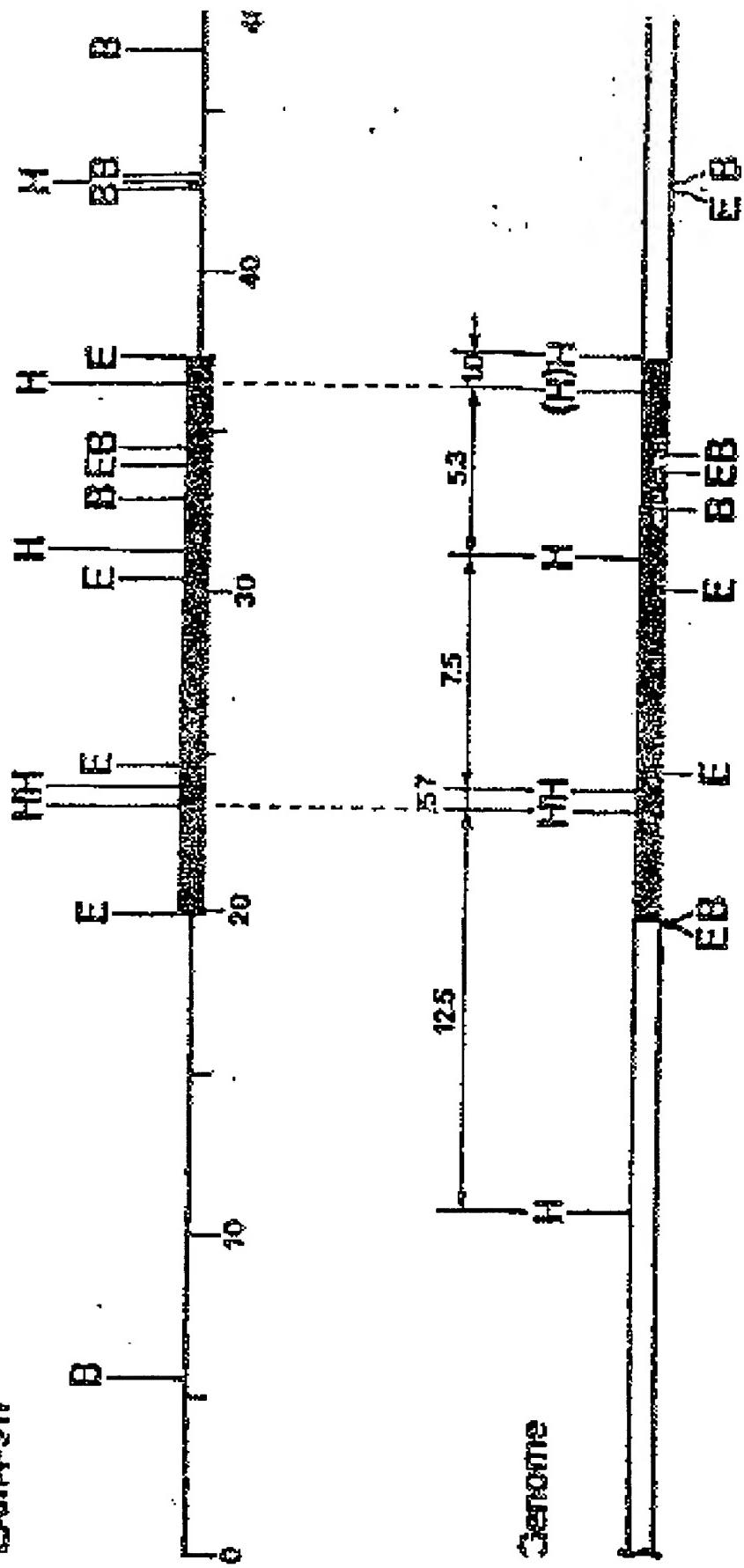
Related Pm - none

Unrelated Pm - BindIII

---

Combined PIC BindIII + HincII = 0.58

LAM4-917



0226288

TABLE 2

TABLE 3

## Linkage Relationships

Loci	Number of informative families	LOD (z) scores at recombinant fractions ( $\theta$ ) off								
		0.01	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40
16q4-817-CF	36 (Cent)	-6.02	0.98	2.84	3.20	2.96	2.44	1.78	1.13	0.55
	3 (HGDMR)	0.14	0.89	0.78	0.76	0.68	0.53	0.39	0.25	0.12
	39 Total	-5.89	1.67	3.63	3.98	3.62	2.97	2.19	1.38	0.67
		$\hat{\theta} = 0.14$ ( $z = 3.95$ ); confidence interval: 0.07–0.25; $\hat{z} = 4.13$ ( $\theta_M = 0.09$ ; $\theta_F = 0.19$ )								
16q4-817-PON	13 (Cent)	4.27	5.01	4.78	4.28	3.66	2.97	2.25	1.61	0.81
		$\hat{\theta} = 0.05$ ( $z = 5.01$ ); confidence interval: 0.01–0.13; $\hat{z} = 5.12$ ( $\theta_M = 0.10$ ; $\theta_F = 0.04$ )								

4/7

Mode of Inheritance Recessive

Risk Summary:

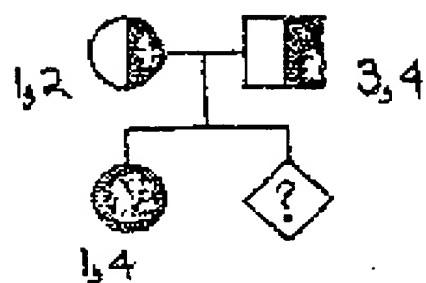
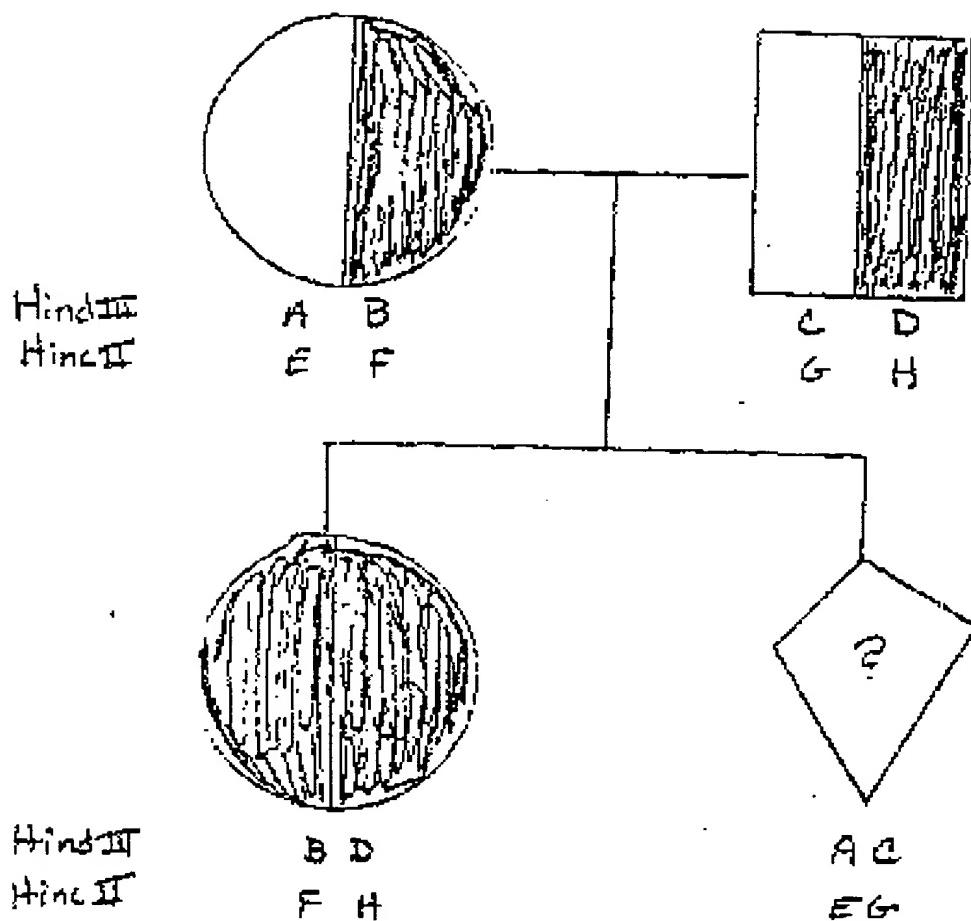
Fully informative RFLP Markers,  $\theta = 0.15$ TABLE 4

TABLE 5

0226288

TABLE 6

	12	60	100	140	180	220	260	300	340	380	420	460	500	540	580	620	660	700	740	780	820	860	900	940	980	1020	1060	1100	1140	1180	1220	1260	1300	1340	1380	1420	1460	1500	1540	1580	1620	1660	1700	1740	1780	1820	1860	1900	1940	1980	2020	2060	2100	2140	2180	2220	2260	2300	2340	2380	2420	2460	2500	2540	2580	2620	2660	2700	2740	2780	2820	2860	2900	2940	2980	3020	3060	3100	3140	3180	3220	3260	3300	3340	3380	3420	3460	3500	3540	3580	3620	3660	3700	3740	3780	3820	3860	3900	3940	3980	4020	4060	4100	4140	4180	4220	4260	4300	4340	4380	4420	4460	4500	4540	4580	4620	4660	4700	4740	4780	4820	4860	4900	4940	4980	5020	5060	5100	5140	5180	5220	5260	5300	5340	5380	5420	5460	5500	5540	5580	5620	5660	5700	5740	5780	5820	5860	5900	5940	5980	6020	6060	6100	6140	6180	6220	6260	6300	6340	6380	6420	6460	6500	6540	6580	6620	6660	6700	6740	6780	6820	6860	6900	6940	6980	7020	7060	7100	7140	7180	7220	7260	7300	7340	7380	7420	7460	7500	7540	7580	7620	7660	7700	7740	7780	7820	7860	7900	7940	7980	8020	8060	8100	8140	8180	8220	8260	8300	8340	8380	8420	8460	8500	8540	8580	8620	8660	8700	8740	8780	8820	8860	8900	8940	8980	9020	9060	9100	9140	9180	9220	9260	9300	9340	9380	9420	9460	9500	9540	9580	9620	9660	9700	9740	9780	9820	9860	9900	9940	9980	10020	10060	10100	10140	10180	10220	10260	10300	10340	10380	10420	10460	10500	10540	10580	10620	10660	10700	10740	10780	10820	10860	10900	10940	10980	11020	11060	11100	11140	11180	11220	11260	11300	11340	11380	11420	11460	11500	11540	11580	11620	11660	11700	11740	11780	11820	11860	11900	11940	11980	12020	12060	12100	12140	12180	12220	12260	12300	12340	12380	12420	12460	12500	12540	12580	12620	12660	12700	12740	12780	12820	12860	12900	12940	12980	13020	13060	13100	13140	13180	13220	13260	13300	13340	13380	13420	13460	13500	13540	13580	13620	13660	13700	13740	13780	13820	13860	13900	13940	13980	14020	14060	14100	14140	14180	14220	14260	14300	14340	14380	14420	14460	14500	14540	14580	14620	14660	14700	14740	14780	14820	14860	14900	14940	14980	15020	15060	15100	15140	15180	15220	15260	15300	15340	15380	15420	15460	15500	15540	15580	15620	15660	15700	15740	15780	15820	15860	15900	15940	15980	16020	16060	16100	16140	16180	16220	16260	16300	16340	16380	16420	16460	16500	16540	16580	16620	16660	16700	16740	16780	16820	16860	16900	16940	16980	17020	17060	17100	17140	17180	17220	17260	17300	17340	17380	17420	17460	17500	17540	17580	17620	17660	17700	17740	17780	17820	17860	17900	17940	17980	18020	18060	18100	18140	18180	18220	18260	18300	18340	18380	18420	18460	18500	18540	18580	18620	18660	18700	18740	18780	18820	18860	18900	18940	18980	19020	19060	19100	19140	19180	19220	19260	19300	19340	19380	19420	19460	19500	19540	19580	19620	19660	19700	19740	19780	19820	19860	19900	19940	19980	20020	20060	20100	20140	20180	20220	20260	20300	20340	20380	20420	20460	20500	20540	20580	20620	20660	20700	20740	20780	20820	20860	20900	20940	20980	21020	21060	21100	21140	21180	21220	21260	21300	21340	21380	21420	21460	21500	21540	21580	21620	21660	21700	21740	21780	21820	21860	21900	21940	21980	22020	22060	22100	22140	22180	22220	22260	22300	22340	22380	22420	22460	22500	22540	22580	22620	22660	22700	22740	22780	22820	22860	22900	22940	22980	23020	23060	23100	23140	23180	23220	23260	23300	23340	23380	23420	23460	23500	23540	23580	23620	23660	23700	23740	23780	23820	23860	23900	23940	23980	24020	24060	24100	24140	24180	24220	24260	24300	24340	24380	24420	24460	24500	24540	24580	24620	24660	24700	24740	24780	24820	24860	24900	24940	24980	25020	25060	25100	25140	25180	25220	25260	25300	25340	25380	25420	25460	25500	25540	25580	25620	25660	25700	25740	25780	25820	25860	25900	25940	25980	26020	26060	26100	26140	26180	26220	26260	26300	26340	26380	26420	26460	26500	26540	26580	26620	26660	26700	26740	26780	26820	26860	26900	26940	26980	27020	27060	27100	27140	27180	27220	27260	27300	27340	27380	27420	27460	27500	27540	27580	27620	27660	27700	27740	27780	27820	27860	27900	27940	27980	28020	28060	28100	28140	28180	28220	28260	28300	28340	28380	28420	28460	28500	28540	28580	28620	28660	28700	28740	28780	28820	28860	28900	28940	28980	29020	29060	29100	29140	29180	29220	29260	29300	29340	29380	29420	29460	29500	29540	29580	29620	29660	29700	29740	29780	29820	29860	29900	29940	29980	30020	30060	30100	30140	30180	30220	30260	30300	30340	30380	30420	30460	30500	30540	30580	30620	30660	30700	30740	30780	30820	30860	30900	30940	30980	31020	31060	31100	31140	31180	31220	31260	31300	31340	31380	31420	31460	31500	31540	31580	31620	31660	31700	31740	31780	31820	31860	31900	31940	31980	32020	32060	32100	32140	32180	32220	32260	32300	32340	32380	32420	32460	32500	32540	32580	32620	32660	32700	32740	32780	32820	32860	32900	32940	32980	33020	33060	33100	33140	33180	33220	33260	33300	33340	33380	33420	33460	33500	33540	33580	33620	33660	33700	33740	33780	33820	33860	33900	33940	33980	34020	34060	34100	34140	34180	34220	34260	34300	34340	34380	34420	34460	34500	34540	34580	34620	34660	34700	34740	34780	34820	34860	34900	34940	34980	35020	35060	35100	35140	35180	35220	35260	35300	35340	35380	35420	35460	35500	35540	35580	35620	35660	35700	35740	35780	35820	35860	35900	35940	35980	36020	36060	36100	36140	36180	36220	36260	36300	36340	36380	36420	36460	36500	36540	36580	36620	36660	36700	36740	36780	36820	36860	36900	36940	36980	37020	37060	37100	37140	37180	37220	37260	37300	37340	37380	37420	37460	37500	37540	37580	37620	37660	37700	37740	37780	37820	37860	37900	37940	37980	38020	38060	38100	38140	38180	38220	38260	38300	38340	38380	38420	38460	38500	38540	38580	38620	38660	38700	38740	38780	38820	38860	38900	38940	38980	39020	39060	39100	39140	39180	39220	39260	39300	39340	39380	39420	39460	39500	39540	39580	39620	39660	39700	39740	39780	39820	39860	39900	39940	39980	40020	40060	40100	40140	40180	40220	40260	40300	40340	40380	40420	40460	40500	40540	40580	40620	40660	40700	40740	40780	40820	40860	40900	40940	40980	41020	41060	41100	41140	41180	41220	41260	41300	41340	41380	41420	41460	41500	41540	41580	41620	41660	41700	41740	41780	41820	41860	41900	41940	41980	42020	42060	42100	42140	42180	42220	42260	42300	42340	42380	42420	42460	42500	42540	42580	42620	42660	42700	42740	42780	42820	42860	42900	42940	42980	43020	43060	43100	43140	43180	43220	43260	43300	43340	43380	43420	43460	43500	43540	43580	43620	43660	43700	43740	43780	43820	43860	43900	43940	43980	44020	44060	44100	44140	44180	44220	44260	44300	44340	44380	44420	44460	44500	44540	44580	44620	44660	44700	44740	44780	44820	44860	44900	44940	44980	45020	45060	45100	45140	45180	45220	45260	45300	45340	45380	45420	45460	45500	45540	45580	45620	45660	45700	45740	45780	45820	45860	45900	45940	45980	46020	46060	46100	46140	46180	46220	46

			Variable Fragment	Constant
	Probe	Enzyme	Length (Kb)	Fragment Length (Kb)
5	S-140	Msp I	Allele 1 - 5.4 Kb Allele 2 - 5.7 Kb	1.7 Kb
	S-002	Msp I	Allele 1 - 4.3 Kb, 2.4 Kb Allele 2 - 2.9 Kb, 2.0 Kb	1.0 Kb
	S-094	Tag I	Allele 1 - 19.0 Kb	none
10			Allele 2 - 14.0 Kb, 7.8 Kb	
	S-158	Tag I	Allele 1 - 6.5 Kb Allele 2 - 5.3 Kb	3.3 Kb, 2.2 Kb
	L4-1033	Msp I	Allele 1 - 12.4 Kb Allele 2 - 10.5 Kb	8.8 Kb
15	S-162	Msp I	Allele 1 - 10.9 Kb Allele 2 - 10.4 Kb	2.3 Kb
	S-157	Msp I	Allele 1 - 9.8 Kb, 3.8 Kb Allele 2 - 2.7 Kb, 1.7 Kb, 1.2 Kb	none
	S-014	Msp I	Allele 1 - 18.0 Kb Allele 2 - 15.0 Kb Allele 3 - 14.5 Kb	8.0 Kb
	S-023	Tag I	Allele 1 - 10.5 Kb Allele 2 - 3.4 Kb	1.0 Kb





Europäisches Patentamt  
European Patent Office  
Office européen des brevets

(11) Publication number:

**O 226 288**  
**A3**

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number: 86307747.5

(51) Int. Cl.4: C 12 Q 1/68, C 12 N 15/00,  
C 12 N 7/00, C 12 P 19/34

(22) Date of filing: 08.10.86

(30) Priority: 09.10.85 US 785798  
29.05.86 US 868351

(71) Applicant: COLLABORATIVE RESEARCH INC., Two Oak Park, Bedford Massachusetts 01730 (US)

(43) Date of publication of application: 24.06.87  
Bulletin 87/26

(72) Inventor: Barker, David F., 45 Kilgore Avenue Medford, Massachusetts 02155 (US)  
Inventor: Braman, Jeffrey C., Alberta Drive Hudson, Massachusetts 01749 (US)  
Inventor: Buchwald, Manuel, 353 Riverdale Avenue Toronto, Ontario M6J184 (CA)  
Inventor: Keller, Helen-Donis, 80 Park Street Brookline, Massachusetts 02146 (US)  
Inventor: Knowlton, Robert G., 30 Shirley Street Lexington, Massachusetts 02173 (US)  
Inventor: Schumm, James W., 27 Moningside Avenue Natick, Massachusetts 0760 (US)  
Inventor: Tsui, Lap-Chee, 170, Elisabeth Street, Hospital Children, Toronto/Ontario M5G 1X8 (CA)

(84) Designated Contracting States: AT BE CH DE ES FR GB  
GR IT LI LU NL SE

(88) Date of deferred publication of search report: 28.06.89 Bulletin 89/26

(74) Representative: Shipley, Warwick Grenville Michael et al, VENNER, SHIPLEY & CO. 368 City Road, London EC1V 2QA (GB)

(54) Means and method of testing for cystic fibrosis based on genetic linkage.

(57) The present invention provides a method of determining presence or absence of one or more cystic fibrosis alleles in a DNA sample taken from an individual having a family genetic background in which family, DNA of ancestors in the family line contains identified cystic fibrosis causing allele genes, said method comprising, restriction digesting a DNA sample to be tested, which DNA sample is from an individual having a family genetic background in which family ancestors of the family have DNA which contains identified cystic fibrosis allele;

apply a probe to said DNA sample which probe hybridizes within less than 50 centimorgans of a cystic fibrosis gene in said family DNA,

and determining whether or not hybridization has taken place in a manner consistent with the presence of one or more cystic fibrosis alleles in said sample.

**EP O 226 288 A3**



European Patent  
Office

EUROPEAN SEARCH REPORT

0226288  
Application Number

EP 86 30 7747

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X	AMERICAN JOURNAL OF HUMAN GENETICS, vol. 36, no. 4 suppl., 1984, page 157S, US; P. WATKINS et al.: "Analysis of amish-mennonite cystic fibrosis pedigree using restriction fragment length polymorphisms" * Whole article * ---	1-30	C 12 Q 1/68 C 12 N 15/00 C 12 N 7/00 C 12 P 19/34
X	AMERICAN JOURNAL OF HUMAN GENETICS, vol. 36, no. 4 suppl., 1984, page 156S, US; L.-C. TSUI et al.: "Mapping the cystic fibrosis locus with polymorphic DNA markers" * Whole article * ---	1-30	
X	JOURNAL OF HUMAN GENETICS, vol. 69, no. 3, 1985, pages 250-254, Springer-Verlag, DE; P. SCRAMBLER et al.: "Linkage studies between polymorphic markers on chromosome 4 and cystic fibrosis" * Abstract * ---	1-30	
X	CYTOGENETICS & CELL GENETICS, vol. 40, nos. 1-4, 1985, pages 587-588, CH; C. BOUCHEIX et al.: "Assignment of the gene coding for the human cell surface antigen CD9(p24) to chromosome 12q1-pter" * Whole article * ---	1-30	C 12 Q
X	CYTOGENETICS & CELL GENETICS, vol. 39, no. 4, 1985, pages 299-301, CH; L.-C. TSUI et al.: "Cystic fibrosis: progress in mapping the disease locus using polymorphic DNA markers. I." * Whole article * ---	1-30	
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	10-03-1989	OSBORNE H.H.	
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone	T : theory or principle underlying the invention		
Y : particularly relevant if combined with another document of the same category	E : earlier patent document, but published on, or after the filing date		
A : technological background	D : document cited in the application		
O : non-written disclosure	L : document cited for other reasons		
P : intermediate document	& : member of the same patent family, corresponding document		



European Patent  
Office

## EUROPEAN SEARCH REPORT

Page 0226288  
Application Number

EP 86 30 7747

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. CL.4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	JOURNAL OF CLINICAL RESEARCH, vol. 32, no. 1, 1984, page 99A; G.M. LEE et al.: "Linkage analysis of cystic fibrosis" * Whole article *	1-30	
Y	WO-A-8 303 260 (PETERSON et al.) * Whole document *	1-30	
Y	EP-A-0 084 796 (CETUS CORP.) * Whole document *	1-30	
Y	GB-A-2 135 774 (ACTAGEN INC.) * Whole document *	1-30	
-----			
TECHNICAL FIELDS SEARCHED (Int. Cl.4)			
-----			
<p>The present search report has been drawn up for all claims</p>			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	10-03-1989	OSBORNE H.H.	
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			

